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Identification of high-lipid producers for biodiesel production from forty-three green algal isolates in China

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Abstract: To identify some desirable algal strains for our future research and/or the production of algae-based biofuel, 43 green algal strains were successfully isolated from Chinese freshwaters, and then incubated in the laboratory bioreactors for the growth and oil accumulation investigations. During a 15 d incubation experiment, the accumulations of their biomass and total lipids, together with the lipid productivities for these green algal strains were systematically investigated and compared. Results indicated that the accumulations of biomass for the 43 algal strains ranged from 0.53 g/L to 6.07 g/L during the experiments, with the highest biomass of 6.07 g/L for green algae *Scenedesmus bijuga*. The lipid content for the tested algal strains varied from 20% to 51% of the dry biomass at the end of cultivation experiments. Green algae *Chlorella pyrenoidosa* was one of the best oil producers based on our investigations, with the total lipid content of 51% of dry biomass. Taking the growth rates and the accumulations of intracellular lipids into the consideration, 10 strains were considered to have significant potential for biofuel applications. In addition, the lipid productivities of the selected strains were further investigated.
Key words: biofuel; microalgae; lipid accumulation

1 Introduction

Due to the limited stocks of fossil fuels and the increasing emission of greenhouse gas, carbon dioxide, into the atmosphere from the combustion of fossil fuels, research has begun to focus on alternative biomass-derived fuels [1−5]. One promising source of biomass for alternative fuel production is microalgae that has the ability to grow rapidly and synthesize and accumulate large amounts (20%−50% of dry mass) of neutral lipid (mainly in the form of triacylglycerol, TAG) stored in cytosolic lipid bodies [2, 6−8]. Some species of diatoms (e.g., *Chaetoceros muelleri*) and green microalgae (e.g., *Chlorella vulgaris*, *Chlorococcum littorale, Botryococcus braunii, Nannochloris*) have been considered to be candidate strains for production of neutral lipids for conversion to various types of biofuels (e.g., biodiesel, kerosene, gasoline) [6, 9−11]. Additionally, microalgae could grow under harsher conditions and has reduced needs for nutrients, so that they could be grown in areas unsuitable for agricultural purposes independently of the seasonal weather changes, without competing for arable land use. Compared with other biomass-derived biofuels, algae-based biodiesel is receiving increasing attentions worldwide in the recent years.

The critical starting point for this process is the identification of suitable algal strains that possess high constituent amounts of total lipids, in general, and neutral lipids, in particular, and/or are capable of rapid accumulation of large quantities of neutral lipids under various culture conditions. In the past decades, many investigations with the aim of screening the oil producers were implemented in north America, Europe, the Middle East, Australia, as well as in many other parts of the world [12−16]. A good example is Aquatic Species Program (ASP) funded by the Department of Energy of United States (US DOE) from 1978 to 1996 representing the most comprehensive research efforts to date on fuels from microalgae [12]. However, few previously isolated strains have gone beyond rather small-scale cultivation

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trials, due to low lipid production under outdoor culture conditions [6, 12]. More algal strains that have high growth potential and high constituent amounts of lipids, and show robustness in various environmental conditions are sought.

In China, only several investigations relating to the algae-based biodiesel could be available at present [17−19]. For the success of algae-based biofuel production technology and commercialization, wide-scale screening of naturally occurring and genetically modified algal strains and mutants for high neutral lipid/oil producers is very crucial in our country. Based on these considerations, the present study is aimed to identify high lipid producers from 43 green algal strains which were obtained from Chinese freshwaters. The growth rate, lipid accumulations as well as the lipid productivities were evaluated systematically. Results of this study will provide important information relating to screening technologies as well as the useful oil producers for future researches on algae-based biodiesel.

2 Experimental

2.1 Algae strains and culture

All the algae strains for the present study were isolated and kept in FACHB (Freshwater Algae Culture Collection, Institute of Hydrobiology, CAS). The detailed information for these algal strains is listed in Table 1.

These algal strains were grown in 200 mL glass tubes (2.5 cm in diameter) with BG11 medium at room temperature, and light intensity was set at 150 μmol/(m^2·s) from the 4th day. The CO_2 concentration was set as 0.5% (by volume) in the bubbling air, and initial cell concentration was set at 0.5 (OD_{680}). During the cultivation period, 15 mL of algal broth was sampled at the 4th, 7th, 9th, 11th, 13th and 15th day, respectively, then was centrifuged (1 500 g, 10 min) using an eppendorf centrifuge. Algal biomass was dried with a vacuum freeze drier (Yamato, Japan). All the lyophilized algal samples were kept at −20 °C prior to lipid extraction and analysis.

2.2 Reagents

All the reagents used in this study were of analytical grade and were purchased from standard sources.

2.3 Growth and lipid accumulation experiment

To measure growth, 5 mL of the algal culture was collected and filtered using a pre-weighed GF-C filter paper (Whatman, USA.) and dried in a 105 °C oven overnight. Algal growth was expressed as the increase in dry algal biomass as a function of time (day) on a volumetric basis. For the determination of lipid content, around 50 mg of the dried algal samples as used for lipid accumulation measurements. For all the growth and lipid accumulation experiment, duplicates were used.

2.4 Gravimetric determination of lipids

Extraction and determination of intracellular lipids were conducted according to the standard protocols in the previous literatures [20−21]. Briefly, freeze-dried algal biomass (50−100 mg) were extracted three times with methanol containing 10% DMSO (volume fraction) for 50 min under stirring. Then the mixture was centrifuged (2 000 g, 10 min), and the supernatant was removed. The residua were re-extracted twice with a mixture of hexane and diethyl ether (1:1, volume ratio) for 30 min. After extractions, hexane and water were added to the combined supernatants, so as to obtain a ratio of 1:1:1:1 (volume ratio) for the above four solvents. The mixture was shaken and then centrifuged for 10 min and 250 g of the upper organic layer was collected. After the water phase was re-extracted twice with a mixture of hexane and diethyl ether (1:1, volume ratio), the organic phases were combined and evaporated to dryness. Then it was redissolved with a small amount of hexane. The lipid solution was transferred into a pre-weighed vial, initially evaporated in a water bath (30 °C) using a rotatory evaporator and then dried under high vacuum. The dried residua were placed under nitrogen and weighed on an electronic microbalance (precision of 0.01 mg, Sartorius, Germany).

2.5 Calculations and statistical analysis

Lipid content (C) was reported as percentage of dry mass (% of DW), while biomass (B) was reported as grams of dried biomass in per liter of culture.

Lipid productivity (P) was reported as milligrams per liter per day using the following equation for calculation:

$$P=1\,000CB/D$$

where P is lipid productivity (mg/(L·d)); C is lipid content (% of DW); B is biomass (g/L); D is cultivation time (d).

3 Results

3.1 Growth measurements

As the starting algae that we used for the experiment was from exponential growth stage, there was no obvious adaptation phase for the algal cultures in this study (data not shown). The stationary phase was observed at the fifth or sixth day from the beginning of the experiment for most of the algae strains. Results indicated from Table 2 that the growth for these algal strains varied dramatically between different species,

Table 1 Origin of 43 algae strains tested

No.	Strain name	Place of origin
1	*Chlorella luteorividis* Strain 1	FACHB Collection, China
2	*Chlorella protothecides* Strain 1	FACHB Collection, China
3	*Chlorella pyrenoidosa* Strain 1	FACHB Collection, China
4	*Chlorella pyrenoidosa* Strain 2	FACHB Collection, China
5	*Chlorella pyrenoidosa* Strain 3	Wuhan, Hubei, China
6	*Chlorella pyrenoidosa* Strain 4	Hubei, China
7	*Chlorella pyrenoidosa* Strain 5	Hubei, China
8	*Chlorella pyrenoidosa* Strain 6	Hunan, China
9	*Chlorella pyrenoidosa* Strain 7	FACHB Collection, China
10	*Chlorella pyrenoidosa* Strain 8	Hunan, China
11	*Chlorella regularis* Strain 1	FACHB Collection, China
12	*Chlorella saccharophila* Strain 1	FACHB Collection, China
13	*Chlorella sorokiniana* Strain 1	FACHB Collection, China
14	*Chlorella sp.* Strain 1	FACHB Collection, China
15	*Chlorella sp.* Strain 2	FACHB Collection, China
16	*Chlorella sp.* Strain 3	FACHB Collection, China
17	*Chlorella sp.* Strain 4	FACHB Collection, China
18	*Chlorella sp.* Strain 5	Wuhan, Hubei, China
19	*Chlorella sp.* Strain 6	Wuhan, Hubei, China
20	*Chlorococcum infusionum* Strain 1	Wuhan, Hubei, China
21	*Chlorella vulgaris* Strain 1	Fujian, China
22	*Chlorella vulgaris* Strain 2	Yunnan, China
23	*Chlorella vulgaris* Strain 3	Yunnan, China
24	*Chlorella vulgaris* Strain 4	Guangdong, China
25	*Chlorella ellipsoidea* Strain 1	Wuhan, Hubei, China
26	*Chlorella ellipsoidea* Strain 2	FACHB Collection, China
27	*Scenedesmus bijuga* Strain 1	FACHB Collection, China
28	*Scenedesmus bijuga* Strain 2	FACHB Collection, China
29	*Scenedesmus dimorphus* Strain 1	FACHB Collection, China
30	*Scenedesmus dimorphus* Strain 2	FACHB Collection, China
31	*Scenedesmus dimorphus* Strain 3	FACHB Collection, China
32	*Scenedesmus obliqnus* Strain 1	Hubei, China
33	*Scenedesmus obliqnus* Strain 2	Hubei, China
34	*Scenedesmus obliqnus* Strain 3	Hubei, China
35	*Scenedesmus obliqnus* Strain 4	Hubei, China
36	*Scenedesmus quadricauda* Strain 1	Donghu Lake, Hubei, China
37	*Scenedesmus quadricauda* Strain 2	FACHB Collection, China
38	*Scenedesmus quadricauda* Strain 3	Donghu Lake, Hubei, China
39	*Scenedesmus sp*. Strain 1	FACHB Collection, China
40	*Scenedesmus sp*. Strain 2	FACHB Collection, China
41	*Scenedesmus sp*. Strain 3	Dianchi Lake, Yunnan, China
42	*Scenedesmus sp*. Strain 4	Donghu Lake, Hubei, China
43	*Scenedesmus sp*. Strain 5	Donghu Lake, Hubei, China

Table 2 Dry biomass, lipid content and lipid productivity of 43 algae strains at the end of cultivation experiment

No.	Algae name	Dry biomass/(g·L^{-1})	Lipid content/%	Lipid productivity/(mg·L^{-1}·d^{-1})
1	*Chlorella luteorividis* Strain 1	0.55±0.21	28.47±3.60	13.74±2.94
2	*Chlorella protothecides* Strain 1	1.32±0.40	31.23±1.09	39.60±5.45
3	*Chlorella pyrenoidosa* Strain 1	1.05±0.54	28.22±1.38	45.41±3.27
4	*Chlorella pyrenoidosa* Strain 2	1.75±0.35	33.15±1.83	49.69±9.97
5	*Chlorella pyrenoidosa* Strain 3	2.23±0.33	31.32±0.09	80.09±17.54
6	*Chlorella pyrenoidosa* Strain 4	3.02±0.40	51.28±0.03	152.55±24.18
7	*Chlorella pyrenoidosa* Strain 5	5.75±0.45	38.52±2.67	194.27±1.56
8	*Chlorella pyrenoidosa* Strain 6	3.97±0.42	52.08±2.37	183.71±23.99
9	*Chlorella pyrenoidosa* Strain 7	1.12±0.06	19.46±1.10	34.13±1.67
10	*Chlorella pyrenoidosa* Strain 8	0.98±0.26	18.67±2.94	15.64±13.88
11	*Chlorella regularis* Strain 1	3.03±0.09	44.36±0.49	126.66±5.53
12	*Chlorella saccharophila* Strain 1	3.88±0.59	45.46±0.34	153.38±18.41
13	*Chlorella sorokiniana* Strain 1	3.37±0.71	28.91±2.28	85.77±21.06
14	*Chlorella sp.* Strain 1	3.18±0.87	20.29±0.46	49.90±15.20
15	*Chlorella sp.* Strain 2	2.87±0.05	24.71±0.34	47.23±0.15
16	*Chlorella sp.* Strain 3	2.05±0.35	19.28±0.95	31.58±9.81
17	*Chlorella sp.* Strain 4	4.62±0.49	26.25±2.89	122.73±9.41
18	*Chlorella sp.* Strain 5	2.43±0.33	20.07±0.17	48.85±12.61
19	*Chlorella sp.* Strain 6	2.38±0.12	28.79±0.40	72.72±22.50
20	*Chlorococcum infusionum* Strain 1	0.82±0.78	20.81±0.86	38.21±4.33
21	*Chlorella vulgaris* Strain 1	1.55±0.49	32.17±2.92	37.57±7.33
22	*Chlorella vulgaris* Strain 2	0.53±0.13	29.36±4.11	18.50±6.16
23	*Chlorella vulgaris* Strain 3	0.67±0.24	24.00±4.49	16.54±6.05
24	*Chlorella vulgaris* Strain 4	1.27±0.00	25.11±2.01	37.84±8.79
25	*Chlorella ellipsoidea* Strain 1	1.58±0.40	27.50±1.97	36.89±6.44
26	*Chlorella ellipsoidea* Strain 2	3.000±0.66	45.35±2.31	116.93±26.16
27	*Scenedesmus bijuga* Strain 1	6.07±0.24	35.24±1.97	178.44±17.00
28	*Scenedesmus bijuga* Strain 2	3.65±1.00	34.10±0.92	123.23±4.97
29	*Scenedesmus dimorphus* Strain 1	3.93±0.18	48.35±2.05	158.19±0.68
30	*Scenedesmus dimorphus* Strain 2	5.87±0.24	43.13±0.42	211.04±10.72
31	*Scenedesmus dimorphus* Strain 3	5.88±0.45	26.35±0.68	121.05±9.93
32	*Scenedesmus obliqnus* Strain 1	5.42±0.02	42.60±1.88	127.86±0.64
33	*Scenedesmus obliqnus* Strain 2	4.47±0.00	42.93±2.28	136.97±7.25
34	*Scenedesmus obliqnus* Strain 3	3.53±0.13	33.82±0.86	99.56±7.18
35	*Scenedesmus obliqnus* Strain 4	4.00±0.14	36.59±0.88	104.59±6.22
36	*Scenedesmus quadricauda* Strain 1	4.22±0.31	27.16±0.15	140.08±12.59
37	*Scenedesmus quadricauda* Strain 2	5.80±0.33	25.26±0.08	141.20±5.78
38	*Scenedesmus quadricauda* Strain 3	3.58±0.94	15.61±0.63	46.76±10.80
39	*Scenedesmus sp*. Strain 1	4.80±0.19	38.36±0.80	131.47±2.40
40	*Scenedesmus sp*. Strain 2	2.33±0.07	37.58±0.08	64.88±9.53
41	*Scenedesmus sp*. Strain 3	3.44±0.03	23.52±1.22	81.66±13.17
42	*Scenedesmus sp*. Strain 4	3.88±0.06	27.69±0.57	75.43±20.99
43	*Scenedesmus sp*. Strain 5	1.88±0.03	21.20±0.13	56.37±4.28

with the accumulations of biomass for the 43 strains ranged from 0.53 g/L to 6.07 g/L. The best biomass producer was *Scenedesmus bijuga* Strain 1 (Maximum biomass was at the 15th day with the value of 6.07 g/L).

3.2 Analysis of lipid content

It was indicated from the results in Table 2, Fig. 1 and Fig. 2 that the tested algal strains showed similar lipid accumulation patterns, that is, the lipid contents began to increase after the lag phase of their growth stage, and reached the maximum values after cultivation for 12 d (Table 2, Fig. 1, Fig. 2). However, the lipid accumulation speed varied significantly depending on the strains.

Our studies also showed that some strains even belonged to the same species (eg. *Chlorella pyrenoidosa*) but presented quite different lipid and biomass accumulations in the experiment period. Results from Table 2 demonstrated that the accumulations of lipid contents for the 43 strains ranged from 20% to 51%. The best oil producer was *Chlorella pyrenoidosa* Strain 4, with the total lipid content of nearly 51% of the dry biomass.

3.3 Calculation of lipid productivity

To identify the desired oil producer, lipid productivities for the 43 algal strains were also investigated and compared in the present study. The highest lipid productivity for each algal strains occurred between the 11th and 13th day, indicating that the optimized harvesting time for these cultures could be selected at the 12th day under laboratory conditions. The

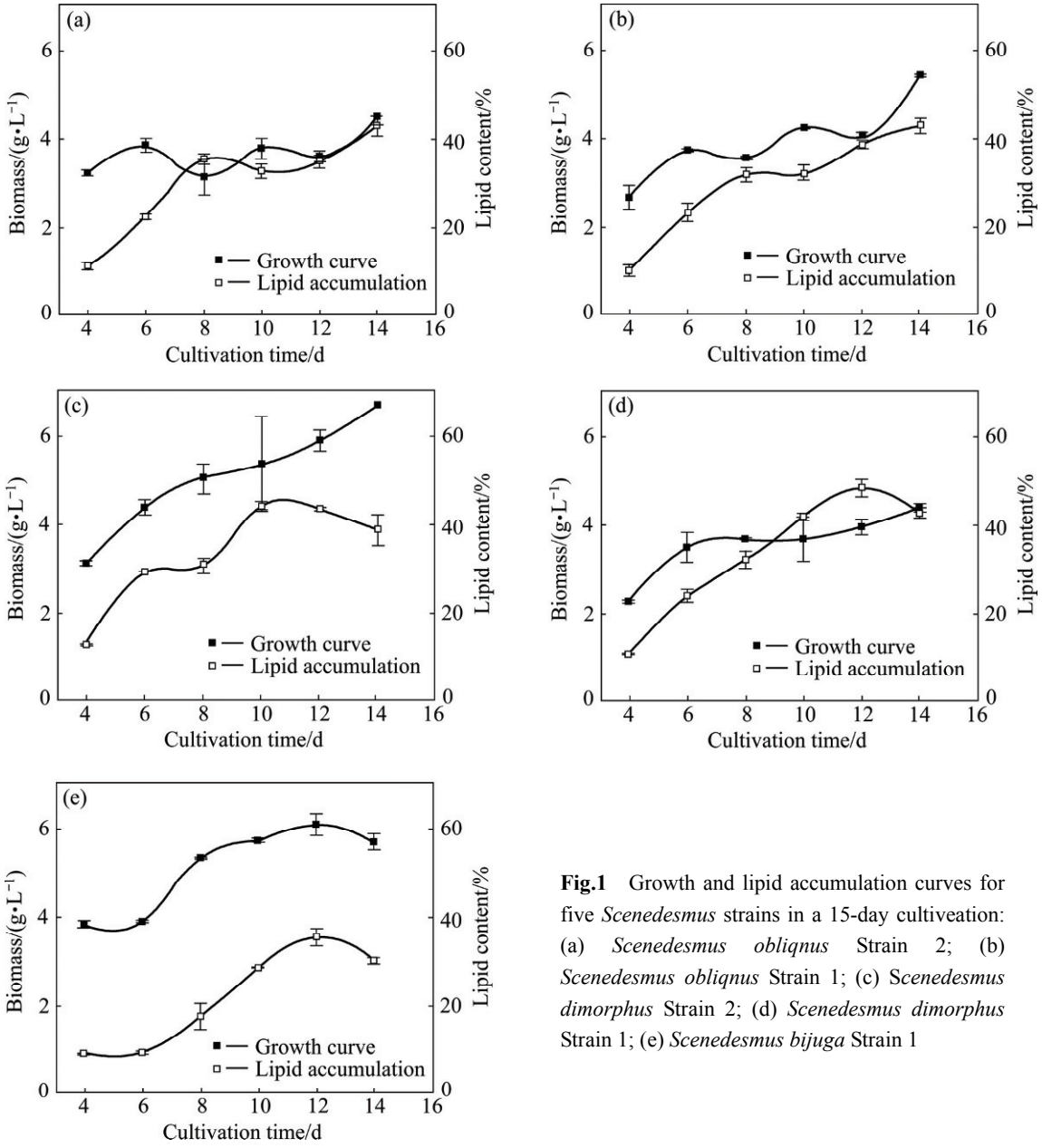

Fig.1 Growth and lipid accumulation curves for five *Scenedesmus* strains in a 15-day cultiveation: (a) *Scenedesmus obliqnus* Strain 2; (b) *Scenedesmus obliqnus* Strain 1; (c) S*cenedesmus dimorphus* Strain 2; (d) *Scenedesmus dimorphus* Strain 1; (e) *Scenedesmus bijuga* Strain 1

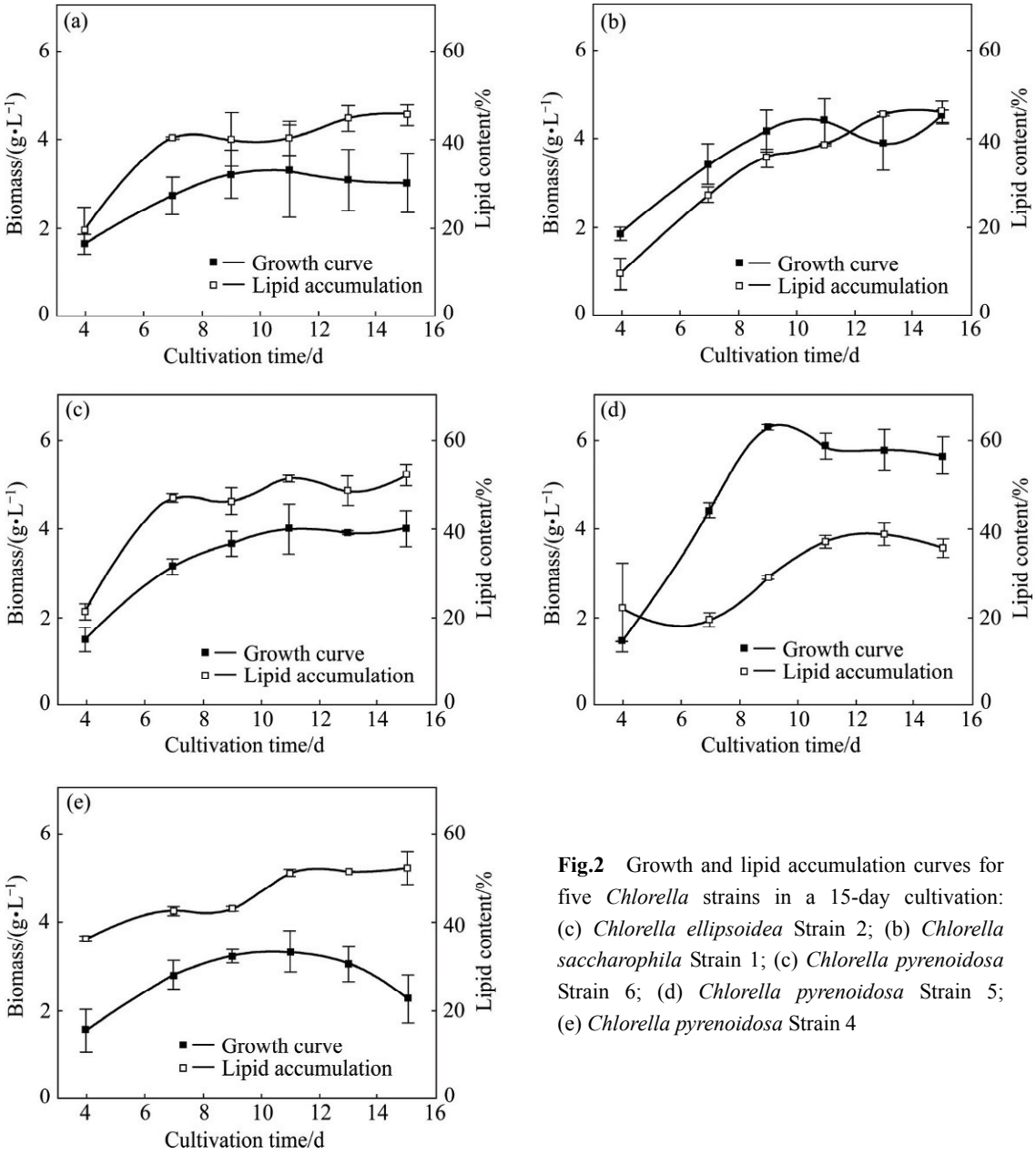

Fig.2 Growth and lipid accumulation curves for five *Chlorella* strains in a 15-day cultivation: (c) *Chlorella ellipsoidea* Strain 2; (b) *Chlorella saccharophila* Strain 1; (c) *Chlorella pyrenoidosa* Strain 6; (d) *Chlorella pyrenoidosa* Strain 5; (e) *Chlorella pyrenoidosa* Strain 4

lipid productivity for these strains ranged from 13 mg/(L·d) to 211 mg/(L·d) during the cultivation period.

3.4 Identification of candidate oil producers

In the first step, 24 algal strains were selected according to their biomass accumulation (>3 g/L). And in the second step, 13 algae strains were selected out of the 24 strains based on total lipid contents (>35% of dry biomass) at the end of experiment. Finally, 10 green algae strains(Fig. 1, Fig. 2) were selected as the candidate oil producers for future outside mass culture after taking the lipid productivities into the consideration. Ten selected algal strains have average total lipid content of over 44% of dry biomass and the average lipid productivity of around 161 mg/(L·d).

4 Discussion

As the starting point for algal biodiesel production, isolating and screening of high neutral lipid-producing microalgae is very crucial to the commercial success of algae-based biofuel production. In the past decades, wide-scale screening of naturally occurring algal strains have been done all over the world [12−16]. However in China, very few investigations relating to the algae-based biodiesel, especially relating to the screening of oleic strains, could be available even at present. In addition, these investigations in China were based only on several specific strains, such as *Chlorella protothecoides, Chlamydomonas reinhardtii* [17−19]. To obtain more

effective strains for the production of algae-based biofuel in our country, we conducted an investigation into the oil and biomass producing characters of 43 green algal strains from Chinese freshwaters in this study to find the high-oil producers.

However, how to select the algal strains and what was the criterion for the good oil-producing strains also puzzled many scientists in the world, as there is still no criterion for the selection process at present. According to our experience in algae-based biofuel research, the expected candidate oil producers should satisfy the following requirements: 1) high growth rate; 2) high cell density at the end of stationary growth stage; 3) high total lipid content; and 4) high lipid productivity. In the previous literatures, total lipid content has been considered an important parameter for the evaluation of oil-producing potentials in the course of seeking the high-quality algal strains [15, 22]. In this study, our identification process is as follows: 1) According to the results presented in Table 2 and Fig. 1(e), we select *Scenedesmus bijuga* Strain 1 as best biomass producer; 2) According to the results indicated in Fig. 2(e), we select *Chlorella pyrenoidosa* Strain 4 as the best oil producer. Fast-growing algae species (eg. *Scenedesmus bijuga* Strain 1, Fig. 1(e)) may not demonstrate that the lipid productivities were higher than those with very high total lipid contents at the end of the experiment. On the other hand, the algae strains with high lipid contents , however, may also improve the efficiency of subsequent biomass processing (*Chlorella pyrenoidosa* strain strain 4, Fig. 2(e)) in oil extraction and refining processes. Based on these considerations, we proposed our criterion that the best lipid producer should show the best combination of biomass productivity and total lipid content. Therefore, lipid content, biomass productivity and their combination to yield lipid productivity were systematically investigated in this study to identify the oil producers. In the present study, we succeeded in identifying 10 green algal strains as candidate oil producers from 43 algal strains based on our criterion of high growth rate, high lipid accumulation and high lipid productivity. The isolated stains from Chinese freshwaters were now kept in FACHB Collection Center as individuals of the fuel-algae bank, which will be very important to the future studies and the production of algae-based biofuel in our country.

In the past years, some algal strains from freshwaters (e.g., *Chlorella, Cylindrotheca, Dunaliella, Isochrysis, Nannochloris, Nannochloropsis, Neochloris, Phaeodactylum, Porphyridium, Schizochytrium, Tetraselmis, Crypthecodinium,*) usually have been reported to have oil contents of 20%−50% of the dry biomass [15, 22−24]. As expected, the total lipid content for all the tested green algae strains in the present study lied within this range. It was reported in the previous studies that the algal strains had lipid content of less than 40% of the dry biomass under nutrient replete conditions; however, much higher lipid content could be obtained when the algae was cultured under nitrogen deficiency conditions. Ten selected algal strains have the average total lipid content of over 44% of dry biomass with the highest value of 51% under the normal culture conditions. The above results demonstrated great potentials of the selected candidate strains for future biodiesel production, as the lipid productions for these strains could be further increased after changing the culture conditions. On the other hand, concerning the lipid productivities, the average value for these ten strains were around 161 mg/(L·d), which were a little higher than the average lipid productivities reported previously with the range of 97 to 160 mg/(L·d) [15, 22−23]. In addition, some species showing high lipid contents but poor biomass productivities were not taken into consideration based our criteria.

Although the algal strains showed good performance in laboratory oil-accumulation experiment, more intensive investigations regarding the oil-producing characters, particularly under field stress conditions (like light stress, nutrient stress, salt stress, etc.) should be implemented in the future. For example, while these algae strains were grown under nutrient shortage conditions, cellular growth generally declined with the overall effect being a decrease of lipid production, although the fraction of lipids increased [10−11, 14, 25]. At the same time, the enhancement of both lipid content and lipid productivity could also be achieved by changing the culture condition a little. As a result, how to improve the lipid productivities of the ten algae strains under nutrient shortage conditions in the field mass culture is very crucial to future studies.

5 Conclusions

1) Three important parameters, total lipid content, biomass productivity and lipid productivity were proposed in this study and were successfully used for the evaluating the ability of algae as biodiesel candidate. In the course of wide-scale screening of 43 naturally occurring algal strains from Chinese freshwaters, 10 green algal strains were selected to be candidate oil producers and showed great potential for neutral lipid production for algae-based biofuel research in China.

2) The present study provides the important information regarding the screening technologies as well as the useful oil producers for future researches and production of algae-based biodiesel in China.

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